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Photoaffinity labelling of melanoma cell MSH receptors

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UV-irradiation at 365 nm of cultured Cloudman S91 mouse melanoma cells in the presence of photoreactive α-MSH analogues induced longlasting receptor stimulation as revealed by the ensuing activation of tyrosinase. Receptor labelling was more efficient with 4-diazirinophenyl and 2-nitro-4-azidophenyl photolabels than with 4-azidophenyl, and was further increased when superpotent [Nle^4,D-Phe^7]-α-MSH was used as ligand. Incubation of B16 melanoma cell membranes with mono-iodinated [Nle^4,D-Phe^7,Trp-(Naps)^9]-α-MSH followed by UV-irradiation at 310–550 nm labelled a single band on SDS-PAGE with a molecular mass ≃ 45 kDa. The displacement curve obtained in a competitive photolabelling experiment paralleled that of the binding assay, demonstrating that the labelling was specific.

Photoaffinity labeling; Carbene; Nitrene; Melanocyte-stimulating hormone receptor; (Cloudman S91 mouse melanoma cell, B16 mouse melanoma cell)

1. INTRODUCTION

α-Melanocyte-stimulating hormone (α-melanotropin) is well known to induce pigment dispersion in melanophores of lower vertebrates and to have neurotrophic and other functions in the nervous system (reviewed in [1]). The hormone also stimulates melanogenesis in mammalian melanocytes and melanoma cells by activating tyrosinase, the rate-limiting enzyme for melanin formation [1]. The hormone acts through a specific membrane receptor which has been characterized extensively by structure-activity and binding studies [1–3].

Photoaffinity labelling has become an important technique for the localization and characterization of peptide hormone receptors and for functional studies [4]. The method has previously been applied to MSH receptors on frog and lizard melanophores, which are irreversibly stimulated after formation of a covalent hormone-receptor complex [5,6], and to the analysis of factors participating in the signal transduction through the membrane [7,8]. The present report describes the labelling of MSH receptors on intact Cloudman S91 mouse melanoma cells using nitrene- and carbene-generating photolabels attached to natural α-MSH and superpotent [Nle^4,D-Phe^7]-α-MSH. Based on these functional studies, the most suitable photoreactive α-MSH derivative was selected for biochemical analysis of the MSH receptor on plasma membranes of B16 mouse melanoma cells.

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Abbreviations: MSH, melanocyte-stimulating hormone (melanotropin); PAGE, polyacrylamide gel electrophoresis; photolabels: Apac, 4-azidophenylacetyl; Naps, 2-nitro-4-azidophenylsulfenyl; Tdbz, 4-(3-trifluoromethyldiazirino)benzoyl

This paper is dedicated to Professor Alfred Pletscher on the occasion of his 70th birthday

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2. MATERIALS AND METHODS

2.1. *Peptides*

The synthesis of the photoreactive analogues of α-MSH and [Nle^4,D-Phe^7]-α-MSH was performed in our laboratory and will be described elsewhere. [Nle^4,D-Phe^7]-α-MSH [9] was obtained from Bachem, Bubendorf (Switzerland) and α-MSH from Ciba-Geigy Ltd, Basel. Mono-iodinated [Nle^4,D-Phe^7,Trp(Naps)^9]-α-MSH was prepared in the dark by the 'equimolar' chloramine T method [1] and purified by HPLC on a C18 reversed-phase column using an exponential gradient of 0.1% TFA/acetonitrile for elution.

2.2. *Bioassay and binding assay*

Mouse melanoma cells (Cloudman S91 and B16-F1) were cultured as described [10]. The biological activity of the photoreactive peptides was determined with the tyrosinase assay using cultured S91 cells [11]. Briefly, 24-well Costar tissue culture dishes, containing an initial density of 3×10^4 cells/well, were incubated at 37°C for 2 days in the presence of 0.2 μCi/well of L-[3′,5′-^{3}H]tyrosine (Amersham; >50 Ci/mmol) and serial dilutions of the peptides. [^{3}H]Tyr was now removed by treating 1 ml supernatant from each well with 50 mg of activated charcoal for 15 min followed by centrifugation. The amount of ^{3}HOH was determined in a β-counter and the relative tyrosinase stimulation was calculated from the ratio of ^{3}HOH formed by stimulated and control groups.

The receptor binding assay was carried out with B16 cell plasma membranes prepared in a similar way as described in [12]. The samples (150–200 μg protein) were incubated in the dark at 15°C for 3 h together with ~0.1 μCi of ^{125}I-labelled [Nle^4,D-Phe^7,Trp(Naps)^9]-α-MSH and different concentrations of α-MSH, using a total of 200 μl assay buffer (50 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$; 1 mM $CaCl_2$; 0.3 mM 1,10-phenanthroline; 1 mM dithiothreitol; 0.1% BSA). The samples were then diluted with 5 ml of ice-cold buffer, centrifuged at 48000 × g for 1 h and the pellets counted in a γ-counter.

2.3. *Photoaffinity labelling*

Photoaffinity experiments with intact S91 cells were performed with the apparatus described in [6], equipped with an interference filter (365 nm), yielding a light intensity of ~15 mW/cm^2. As opposed to the normal tyrosinase assay, the cells were exposed to 10^{-7} M peptide solutions for only 1 h in the dark and then irradiated for 1 min at 4°C (4 wells at a time). Unbound hormone was removed by thorough washing of the cells which were then incubated with [^{3}H]Tyr for 2 days as described above.

Receptor labelling of B16 cell plasma membranes was carried out as described above for the binding assay, except that BSA was replaced by thyroglobulin. After preincubation for 3 h at 15°C, the samples were UV-irradiated for 3 min at 4°C using the whole 310–550 nm spectrum (~180 mW/cm^2). The samples were then diluted with 5 ml ice-cold buffer and centrifuged at 48000 × g for 1 h. The pellets were solubilized at 22°C by treatment with urea (50%)/Triton X-100/solubilization mixture (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 5% mercaptoethanol, 0.001% bromophenol blue [13]) using a 25:1:15 ratio. The proteins were separated by electrophoresis on 11% SDS polyacrylamide slab gels. The autoradiograms were quantified with a gel scanner and by reflectometry.

3. RESULTS

Photoreactive α-MSH analogues containing Apac in position 1 or Naps in position 9 are full and equipotent agonists which retain 50% MSH activity in the tyrosinase assay (table 1). Attachment of the more lipophilic Tdbz group to Ser^1 decreases the potency to about 30% of the parent peptide. [Nle^4,D-Phe^7]-α-MSH is 120-fold more active than α-MSH, and the Naps^9 derivative still retains a 25-fold higher activity than α-MSH, thus representing the most potent MSH photolabel.

Photoaffinity labelling of Cloudman S91 cells in tissue culture demonstrates that a 1-h incubation of the cells with photoreactive MSH analogues in the dark followed by a 1-min UV-irradiation phase at 365 nm and several washes is sufficient to induce marked stimulation of tyrosinase (fig.1). α-MSH (**1**) without photolabel produces only a marginal effect. [Apac-Ser^1]-α-MSH (**2**) induces a 6.5-fold increase of tyrosinase activity, [Trp(Naps)^9]-α-MSH (**3**) a 10.5-fold and [Tdbz-Ser^1]-α-MSH (**4**) a 13-fold increase as compared to

Table 1

Structure and biological activity of photoreactive α-MSH analogues

Compound[a]	Photolabel	Tyrosinase stimulation EC_{50}[b] (M)
1 α-MSH	–	2.5×10^{-9}
2 [Apac-Ser[1]]-α-MSH	4-azidophenylacetyl	5×10^{-9}
3 [Trp(Naps)[9]]-α-MSH	2-nitro-4-azidophenylsulfenyl	5×10^{-9}
4 [Tdbz-Ser[1]]-α-MSH	4-(3-trifluoromethyldiazirino)benzoyl	8×10^{-9}
5 [Nle[4],D-Phe[7]]-α-MSH	–	2×10^{-11}
6 [Nle[4],D-Phe[7],Trp(Naps)[9]]-α-MSH	2-nitro-4-azidophenylsulfenyl	1×10^{-10}

[a] Apac and Tdbz are attached to the N-terminus of the molecule, replacing the acetyl group. Naps is linked to the 2′-position of the indole moiety of tryptophan

[b] Cloudman S91 mouse melanoma cells

non-irradiated controls. The [Nle[4],D-Phe[7]]-α-MSH analogue (**5**), which displays exceptional receptor affinity, has similar characteristics to **4**; its photoreactive derivative **6** produces the most conspicuous receptor stimulation because of the combination of very high initial receptor binding and covalent linkage to the receptor.

Incubation of plasma membranes from B16 mouse melanoma cells with ^{125}I-labelled [Nle[4],D-Phe[7],Trp(Naps)[9]]-α-MSH followed by UV-irradiation, SDS-PAGE and autoradiography demonstrated that the radioactivity was localized in a single band with a molecular mass ≈45 kDa (fig.2). (B16 cells were preferred for this type of experiment because of a 5-fold higher receptor concentration than in S91 cells; Siegrist, W. and Eberle, A.N., unpublished.) When the samples for SDS-PAGE were prepared as described in [13] for

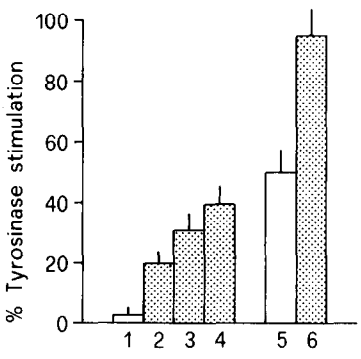

Fig.1. Increase in tyrosinase activity after MSH-receptor labelling of intact Cloudman S91 melanoma cells with different α-MSH peptides, as compared to non-irradiated controls treated with the same ligand. Peptides **1**–**6** are described in table 1. Each bar is the mean of 8 determinations ± SE.

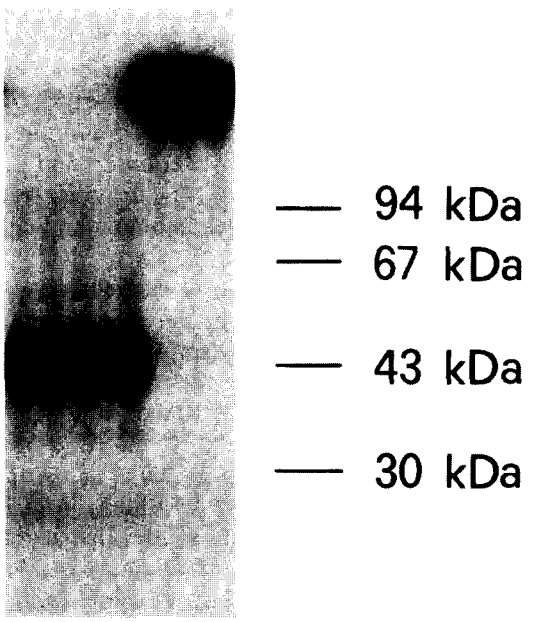

Fig.2. Autoradiogram of an SDS-PAGE separation of labelled B16 membrane proteins. Left lanes: treatment of the membrane pellets as described in section 2; right lanes: aggregated MSH-receptor after heating of the pellets in solubilization mixture.

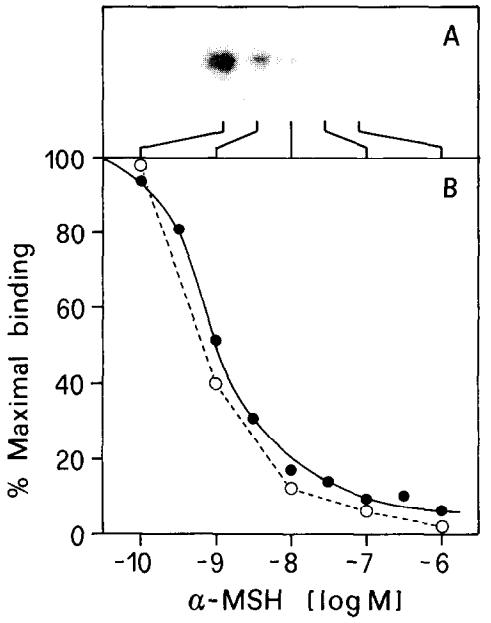

Fig.3. (A) Autoradiogram of a competitive labelling experiment using a constant amount of ^{125}I-labelled [Nle^4,D-Phe^7,Trp-(Naps)^9]-α-MSH and different concentrations of α-MSH. (B) Quantitative analysis of the autoradiogram (○) and comparison with the displacement curve in the binding assay using photo-MSH as tracer (●).

the analysis of melanoma cell phosphoproteins (→solubilization mixture + heating), the radioactivity was found at a much higher molecular mass due to aggregation of the labelled MSH receptor.

The specificity of the MSH receptor labelling was assessed by comparing the labelling pattern of a competitive photoaffinity experiment with the binding characteristics of the radioactive photoligand in a normal binding assay (fig.3). The parallel displacement curves in both types of experiment demonstrate that the labelling of the 45 kDa protein reflects specific binding of α-MSH to melanoma cells.

4. DISCUSSION

The biological response of melanoma cells (i.e. tyrosinase activation and melanin formation) is slow and therefore functional studies with cultured cells are more difficult to perform than with the rapidly responding melanophores. In addition, the cells are very sensitive to UV-light which must be filtered ($\lambda \geq 365$ nm) and the irradiation time kept as short as possible (Eberle, A.N., unpublished). The diazirinophenyl photolabel, originally developed by Brunner et al. [14] for membrane labelling, is particularly useful for functional studies with intact melanoma cells because it is more readily photolysed at longer wavelengths than the 4-azidophenyl group, resulting in a higher labelling efficiency. The lipophilic character of [Tdbz-Ser^1]-α-MSH may however give rise to non-specific labelling (Eberle, A.N., unpublished).

For the photoaffinity labelling of MSH receptors on plasma membranes, the wavelength of the UV-light is less critical than the affinity of the peptide ligand. Therefore, the most potent α-MSH analogue, [Nle^4,D-Phe^7]-α-MSH [9], was chosen and a 2-nitro-4-azidophenyl group introduced into Trp^9 using the Naps-Cl reagent [15]. This peptide was not only more potent than α-MSH but also proved to retain excellent bioactivity after iodination.

There is good evidence that the 45 kDa membrane protein labelled by the radioactive MSH photolabel is the MSH receptor or part of it: (i) labelling of intact melanoma cells produced a biological response, indicating that covalent MSH-receptor crosslinking did take place; (ii) non-irradiated MSH photolabel was specifically and reversibly bound by plasma membranes; (iii) the MSH photolabel was only found in the 45 kDa band on SDS-PAGE and its covalent insertion was competitively inhibited by increasing concentrations of α-MSH, in parallel to the curve obtained in the binding assay. Preliminary studies (Solca, F. and Eberle, A.N., unpublished) indicate that the 45 kDa protein is a glycoprotein; a varying degree of glycosylation could explain the relatively broad band on SDS-PAGE.

The 'small' size of 45 kDa for the MSH receptor (subunit) is not exceptional as the recent characterization of receptor systems for other peptide hormones revealed molecular mass values of 30 kDa (renal V_2 vasopressin receptor) [16], 30 and 38 kDa (hepatic V_1 vasopressin receptor) [17] and 65 kDa (angiotensin II receptor) [18].

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REFERENCES

[1] Eberle, A.N. (1988) The Melanotropins; Chemistry, Physiology and Mechanism of Action, Karger, Basel.
[2] Hruby, V.J., Wilkes, B.C., Cody, W.L., Sawyer, T.K. and Hadley, M.E. (1984) in: Peptide and Protein Reviews (Hearn, M.T.W. ed.) vol.3, pp.1–64, Dekker, New York.
[3] Eberle, A.N., De Graan, P.N.E., Baumann, J.B., Girard, J., Van Hees, G. and Van de Veerdonk, F.C.G. (1985) in: Pigment Cell 1985 (Bagnara, J. et al. eds) pp.191–196, University of Tokyo Press, Tokyo.
[4] Eberle, A.N. and De Graan, P.N.E. (1985) Methods Enzymol. 109, 129–156.
[5] De Graan, P.N.E. and Eberle, A.N. (1980) FEBS Lett. 116, 111–115.
[6] Eberle, A.N. (1984) J. Receptor Res. 4, 315–329.
[7] De Graan, P.N.E., Eberle, A.N. and Van de Veerdonk, F.C.G. (1981) FEBS Lett. 129, 113–116.
[8] Eberle, A.N. and Girard, J. (1985) J. Receptor Res. 5, 59–81.
[9] Sawyer, T.K., Sanfilippo, P.J., Hruby, V.J., Engel, M.H., Heward, C.B., Burnett, J.B. and Hadley, M.E. (1980) Proc. Natl. Acad. Sci. USA 77, 5754–5758.
[10] Siegrist, W. and Eberle, A.N. (1986) Anal. Biochem. 159, 191–197.
[11] Fuller, B.B., Lunsford, J.B. and Iman, D.S. (1987) J. Biol. Chem. 262, 4024–4033.
[12] Gerst, J.E., Sole, J., Mather, J.P. and Salomon, Y. (1986) Mol. Cell. Endocrinol. 46, 137–147.
[13] De Graan, P.N.E., Brussaard, A.B., Gamboni, G., Girard, J. and Eberle, A.N. (1987) Mol. Cell. Endocrinol. 51, 87–93.
[14] Brunner, J., Senn, H. and Richards, F.M. (1980) J. Biol. Chem. 255, 3313–3318.
[15] Muramoto, K. and Ramachandran, J. (1981) Biochemistry 20, 3376–3380.
[16] Fahrenholz, F., Kojro, E., Müller, M., Boer, R., Löhr, R. and Grzonka, Z. (1986) Eur. J. Biochem. 161, 321–328.
[17] Fahrenholz, F., Boer, R., Crause, P. and Tóth, M. (1985) Eur. J. Biochem. 152, 589–595.
[18] Guillemette, G., Guillon, G., Marie, J., Balestre, M.N., Escher, E. and Jard, S. (1986) Mol. Pharmacol. 30, 544–550.